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Karen Van Hoecke[a,*], Karel AC De Schamphelaere[a], Sonia Ramirez[b], Paul Van der Meeren[c], Guy Smagghe[d] and Colin R. Janssen[a].

[a]Laboratory of Environmental Toxicology and Aquatic Ecology, Faculty of Bioscience Engineering, Ghent University, Jozef Plateaustraat 22, B-9000, Gent, Belgium.

[b]Centre for BioNano Interactions, School of Chemistry and Chemical Biology, University College Dublin, Belfield, Dublin 4, Ireland.

[c]Particle and Interfacial Technology Group, Faculty of Bioscience Engineering, Ghent University, Coupure Links 653, B-9000 Gent, Belgium.

[d]Laboratory of Agrozoology, Faculty of Bioscience Engineering, Ghent University, Coupure Links 653, B-9000 Gent, Belgium

* Corresponding author:

Karen Van Hoecke

Phone: +32 – 9 – 264 3710

Fax: +32 – 9 – 264 3766

Email: karen.vanhoecke@ugent.be

Abstract – Silica nanoparticles (NPs) belong to the industrially most important NP types. In a previous study it was shown that amorphous SiO_2 NPs of 12.5 and 27.0 nm are stable in algal growth inhibition assays and that their ecotoxic effects are related to NP surface area. Here, it was hypothesized and demonstrated that an alumina coating completely alters the particle-particle, particle-test medium and particle-algae interactions of SiO_2 NPs. Therefore, stability and surface characteristics, dissolution, nutrient adsorption and effects on algal growth rate of both alumina coated SiO_2 NPs and bare SiO_2 NPs in OECD algal test medium in function of pH (6.0-8.6) and natural organic matter (NOM) contents (0-12 mg C/l) were investigated. Alumina coated SiO_2 NPs aggregated in all media and adsorbed phosphate depending on pH and NOM concentration. On the other hand, no aggregation or nutrient adsorption was observed for the bare SiO_2 NPs. Due to their positive surface charge, alumina coated SiO_2 NPs agglomerated with *Pseudokirchneriella subcapitata*. Consequently, algal cell density measurements based on cell counts were unreliable and hence fluorescent detection of extracted chlorophyll was the preferred method. Alumina coated SiO_2 NPs showed lower toxicity than bare SiO_2 NPs at concentrations $\geq$ 46 mg/l, except at pH 6.0. At low concentrations, no clear pH effect was observed for alumina coated SiO_2 NPs, while at higher concentrations phosphate deficiency could have contributed to the higher toxicity of those particles at pH 6.0-6.8 compared to higher pH values. Bare SiO_2 NPs were not toxic at pH 6.0 up to 220 mg/l. Addition of NOM decreased toxicity of both particles. For SiO_2 NPs the 48 h 20 % effect concentration of 21.8 mg/l increased 2.6-21 fold and a linear relationship was observed between NOM concentration and effective concentrations. No effect was observed for alumina coated SiO_2 NPs in presence of NOM up to 1000 mg/l. All experiments point out that the alumina coating completely altered NP interactions. Due to the difference in surface composition the SiO_2 NPs, which had the smallest surface area, were more toxic to the alga than the alumina

coated SiO_2 NPs. Hence, surface modification can dominate the effect of surface area on toxicity.

Keywords – SiO_2, coating, nanoparticles, algae, NOM

1. INTRODUCTION

Scientific research involving nanotechnology has grown exponentially (Braun et al., 1997). This led to the development of engineered nanoparticles (NPs) and nanoparticle containing consumer products with improved performances compared to non-nanotechnology based products. The special feature of nanoparticles is their small size, between 1 and 100 nm in two or three dimensions (ASTM, 2006), and related high amount of specific surface area, which enhances their reactivity (Oberdörster et al., 2005). Due to production, use and disposal of NPs or NP containing products, they are expected to be released into the environment. Therefore, concerns about the potential environmental risks posed by NPs have been raised (Colvin et al, 2003). In addition to the establishment of effect concentrations for NPs, other research priorities were to assess the importance of general NP characteristics, like size, surface area, chemical composition, solubility and aggregation behaviour for their (eco)toxicity (Tran et al., 2005). An often overlooked parameter is the presence of a surface coating. To our knowledge, no ecotox studies have systematically investigated the influence of a surface coating on the physical and toxicological properties of NPs.

In this study, we describe the differential characteristics and effects of a bare SiO_2 and an alumina coated SiO_2 NP. Silicon dioxide (SiO_2) nanoparticles are among the most important industrially engineered NPs (Rittner, 2003). They are used in paints and coatings for an improved rheology, attachment and scratch-resistance and in printer toners they serve as anti-binder (Mizutani et al., 2006; Zappa et al., 2009). Furthermore, these NPs are used in chemical or mechanical polishing processes, among which dental polishing to prevent tooth caries (Gaikwad et al., 2008). Other medical applications are the use of SiO_2 NPs as carrier for therapeutic agents or for diagnostic purposes (Wang et al, 2006; Zhang et al., 2008). Silica nanoparticles can act as a binding site for negatively charged ions when an

alumina coating is applied to their surface (Li and Stöver, 2008). Because of their numerous applications and high production volumes, both silica and alumina were included in the list of representative nanomaterials adopted by OECD's working party on manufactured nanomaterials (OECD, 2010).

In a previous study, we demonstrated that bare SiO_2 NPs were stable in algal test medium and that their ecotoxicity was related to their surface area. Furthermore, the NPs were found attached to the algal cell wall and toxicity was not due to particle dissolution (Van Hoecke et al., 2008). However, in the present study it was hypothesized that an alumina coating might alter the particle-particle, particle-alga and particle-test medium interactions of SiO_2 NPs. Therefore, NP suspension stability was investigated in test media with varying pH and natural organic matter (NOM) concentration. In algal growth inhibition assays with *Pseudokirchneriella subcapitata* the influence of both parameters on SiO_2 and alumina coated SiO_2 NP toxicity was assessed. In order to prevent erroneous conclusions due to particle-alga interactions, algal growth was measured based on cell density (measured directly with a cell counter) or on chlorophyll contents relative to a standard series of non exposed cells with known cell densities. Finally, we investigated here the appearance of a shading effect, which is the inhibition of algal growth due to light limitation caused by absorption or scattering by the NPs, and particle-test medium interactions, i.e. the dissolution of NPs and nutrient adsorption by the NPs. To exclude the contribution of any impurities to the toxic effects, NP suspensions had been dialyzed with deionized water prior to the experiments.

2. MATERIALS AND METHODS

2.1. Chemicals and nanoparticle specifications

All chemicals were of pro analytical grade supplied by VWR International (Leuven, Belgium). An Al^{3+} standard was purchased at Sigma Aldrich (Bornem, Belgium) (No. 39435). Natural organic matter (NOM) was sampled from 'Le puisseau de St. Martain', a creek in Bihain, Belgium, using a portable reverse-osmosis based device (PROS/2) in order to concentrate NOM as described by Serkiz and Perdue (1990) and Sun et al. (1995). The sampling procedure was described in more detail in De Schamphelaere et al. (2003).

Commercially available LUDOX® aqueous colloidal silica suspensions were obtained from Sigma-Aldrich, i.e. the negatively charged LUDOX CL-X SiO_2 nanoparticles (NPs) (No. 420891) and the positively charged alumina coated SiO_2 NPs LUDOX CL (No. 420883). The core material of the latter NP consists of amorphous SiO_2 to which an approximately 1 nm layer coating of alumina was deposited (Vo et al., 2007). The alumina coating arose through adsorption of Al^{3+} ions to the negatively charged SiO_2 NP surface. With increasing pH, more Al ions adsorp to the surface, which precipitate as aluminium hydroxide ($Al(OH)_3$), forming a solid coating onto the SiO_2 NPs (Kuan et al., 2000). Van der Meeren et al. (2004) observed a decreasing surface charge from pH 5 on and a switch in zetapotential from positive to negative at pH 2.5. The latter observation suggests that at low pH values, the solid coating dissolves and Al^{3+} ions adsorbed to the SiO_2 NP surface can be substituted by protons (H^+) (James and Healy, 1972). Hence, the first iso-electric point (IEP) at pH 2.5 is the point of zero charge of the bare SiO_2 NP core.

Except for the first experiment in which the toxicity of dialyzed and non dialyzed suspensions was compared, dialyzed aqueous suspensions of both silica products were used for further experiments. The dialysis of the NP suspensions was performed using dialysis membranes (12.4 kDa) from Sigma Aldrich (No. D9652). The sample was placed inside the dialysis tubes and submerged in 5 l of Milli-Q water. The sample was left for a minimum of 4 h each time and the water was renewed four times, since it was observed that the

conductivity of dialysis water did not further decrease after 4 dialysis cycles (supporting information, Figure S1). Once the dialysis was complete, the concentration of the stock suspension was determined gravimetrically after freeze drying 1 ml of suspension in a freeze dryer overnight at -60 °C and 0.1 bar pressure. As a consequence, any impurities possibly present in the industrial product could be removed. Nitrogen gas adsorption experiments were used to measure the specific surface area of both particle types using a Tristar 3000 BET instrument (Micrometrics, Norcross, GA, USA). Specific surface area of the LUDOX CL-X SiO_2 NPs was 102 m^2/g, while the LUDOX CL alumina coated SiO_2 NPs represented a specific surface area of 203 m^2/g. Primary particle sizes calculated from these measurements were 22 and 11 nm, respectively.

Nanoparticle suspensions in OECD ecotoxicity test medium were prepared from either the original or dialyzed stock by spiking the silica NPs dropwise into the test media under continuous stirring. Where appropriate, NOM and/or buffer solutions (final concentration of 3.6 mM buffer) were added to the medium and pH was adjusted to the desired value using 1 M NaOH or HCl solutions before spiking the NPs. To maintain a pH value of 6.0 2-(N-Morpholino)ethanesulfonic acid (MES) buffer was used. Media pHs 6.6 and 7.6 were stabilized with 3-(N-Morpholino)propanesulfonic acid (MOPS) buffer and pH 8.6 required 2-(Cyclohexylamino)ethanesulfonic acid (CHES) buffer.

2.2. Suspension characterization

Particle size distributions of both NP types in all test media were analyzed using dynamic light scattering (DLS) four days after suspension preparation as described in Van Hoecke et al. (2008). A concentration of 2 g/l silica NPs and 460 mg/l alumina coated silica NPs was used for the DLS analysis with a PCS 4700 SM (Malvern Instruments, Worcestershire, UK) equiped with a 5 mW HeNe laser. Scattered light was detected under an angle of 150 ° and

data was processed by a 7032 CN correlator (Malvern Instruments). Non negative least squares analysis was used to determine the particle size distribution, whereas the harmonic intensity weighed average hydrodynamic diameter, also referred to as the Z-average diameter (Zave), was obtained by cumulant analysis option of the automeasure software (Malvern Instruments). The zetapotential in OECD medium at various pH values was determined using a Zetasizer 3000 HSA (Malvern Instruments, Worcestershire, UK). During an algal growth inhibition test, pH was monitored each day using a pH electrode (P407, Consort, Turnhout, Belgium) and adjusted from 0.2 pH units deviation on. The media at pH 8.6 were checked twice a day. Natural organic matter concentrations were monitored in a buffer free replicate in OECD algal test medium incubated under experimental conditions and measured with A TOC-500 (Shimadzu, Duisburg, Germany) carbon analyzer.

2.3. Algal culturing and growth inhibition tests

The alga *Pseudokirchneriella subcapitata* (Korshikov) Hindak was obtained from the Culture Collection of Algae and Protozoa (CCAP 278/4, Oban, Scotland) and subcultured in the laboratory. The culture medium consisted of ES-medium (Provasoli, 1966) at 1/2 strength which was added to carbon filtered aerated tap water, supplemented with 1.4 mg/l $FeSO_4.7H_2O$, 15 mg/l $NaH_2PO_4.2H_2O$, 150 mg/l $NaNO_3$ and 2.35 mg/l $MnCl_2.4H_2O$. Each week, cultures were visually inspected for contamination using a light microscope. Four days prior to the start of a growth inhibition experiment, a new algal culture was prepared and allowed to grow on a shaking table at 20 ± 1 °C in continuous light (70 μE/(m^2.s)).

The 72 h algal growth inhibition experiments were conducted in accordance with OECD guideline No. 201 (OECD, 2006). Prior to the start of a test, all test concentrations ranging between 4.6 and 1000 mg/l were equilibrated at 25 °C overnight. For each test

concentration three replicates and one background correction (no algae added) were included. The replicates were inoculated with 10^4 algal cells/ml. During the 72 h test, all flasks were incubated at a temperature of 25 °C under continuous illumination (70 μE/(m^2.s)) and were shaken manually three times a day. Every day, the algal cell density was measured in all replicates based on both an electronic cell count and chlorophyll contents. In the former method, a sample was introduced into a cell counter (Beckman Coulter Counter, Gent, Belgium) and the number of cells/ml was determined. The latter method consisted of adding 3 ml extraction mixture (dimethylsulfoxide (DMSO):acetone 1:1) to a 0.75 ml sample, vortexing and allowing to stand in the dark for 20 minutes to extract the chlorophyll. Chlorophyll fluorescence was recorded with a spectrophotometer (LS50B, Perkin Elmer, Zaventem, Belgium) in a 1 cm quartz cuvette at a wavelength of 671 nm, using an excitation wavelength of 431 nm (Mayer et al., 1997). Preliminary research indicated that the presence of both NP types in the concentrations used in the experiments did not affect the fluorescent signal of chlorophyll (results not shown). The algal cell density was determined against a concentration series of algal cells in OECD medium that had been inoculated with the same culture and extracted under identical conditions. Each day, a new algal concentration series was prepared and analyzed. The average specific growth rate μ (1/d) was calculated as the slope of a linear regression of the natural logarithm of the measured cell density (corrected for background or blank) versus time. Data analyzed after 48 h were reported here, because experiments performed at pH 6.0 and 8.6 did not always meet the validity criteria prescribed by the OECD when analyzed at 72 h. Where possible, data analyzed after 72 h is given in the supplementary information section. A log-logistic or modified log-logistic concentration-response curve was fitted to the toxicity data with Statistica 6.0 (Statsoft, Tulsa, OK).

Additionally, the occurrence of a shading effect, i.e. decrease in algal growth due to limitation in the light availability caused by the nanoparticles, was assessed separately using 1000 mg/l NP suspensions in OECD medium at pH 7.4. The experimental approach consisted of two 96 well plates, i.e. an opaque plate fixed on top of a white plate. Basically, the chlorophyll contents upon spatially separating particles and algal cells was compared to the chlorophyll contents when algal cells and NPs were added to the same well. The same set-up was formerly described in Hund-Rinke et al. (2006) and in Van Hoecke et al. (2009).

2.4. Assessment of interactions between nanoparticles and test medium

In NOM free medium, two types of possible interactions between particles and test media were investigated, i.e. partial dissolution and adsorption of the nutrients ammonium (NH_4^+) and phosphate (PO_4^{3-}) to the NP surface. Nanoparticle suspensions were prepared and incubated in such a way that experimental conditions were identical to those during the 72 h algal gowth inhibition tests, but without addition of algal cells. After 48 h, NPs were removed from the suspensions. To this end, the alumina coated SiO_2 NP suspensions were first centrifuged at 2000x*g* for 15 minutes in a swinging-bucket centrifuge (IEC centra-8 centrifuge, International Equipment Co, Needham, MA) to sediment large aggregates. Then, 20 ml samples were forced through 10 kDa ultrafilters with polyethersulfon membrane (Vivaspin 20, Sartorius, Goettingen, Germany) by a 10 minute centrifugation at 2000x*g*. The filtrate was used for further analysis. The ultrafilter performance, i.e. binding of analytes to the filter material or leaching of analytes from either the filter material or from particles retained by the filter, was checked by including additional controls after every 80 ml run through the filter. More specifically, to check for possible binding of analytes to the filter material, standard OECD medium controls were filtered and its filtrate included in the colorimetric analysis. Leachage of analytes from the filter material or from the retained particles on the filter into subsequent samples was checked by including

deionized water controls. Furthermore, the retention of Al by the filters was assessed at various pH values with 0.5 mg Al/l standards in OECD medium without Fe and EDTA. The concentration of reactive silica, aluminum, NH_4^+ and PO_4^{3-} was colorimetrically assessed with an Aquamate spectrophotometer (Thermo Electron Corporation, Waltham, MA, US). Reactive silica was complexed with ammonium molybdate and subsequently reduced with sodium sulfite, based on ASTM procedure D859-00 (ASTM, 2000). The absorbance of the blue complex was recorded at 700 nm in a 1 cm cuvette. Aluminum concentration was measured in a 2 cm cuvette using a commercial analysis kit No 1.14825.0001 from Merck KgaA (Darmstadt, Germany). In addition, chemical speciation of aluminum in OECD medium at various pH values was estimated with Visual MINTEQ ver. 2.51 program (CEAM, EPA, US). Colorimetric analysis of NH_4^+ and PO_4^{3-} was performed in a 1 cm cuvette using commercial analysis kits (no. 1.14848.0001 and no. 1.14752.0001).

In NOM containing buffer free medium at pH 7.4, the adsorption of both NOM and phosphate to the surface of alumina coated SiO_2 NPs was investigated. Therefore, after 48 h incubation under test conditions, 0, 4.6, 46 and 460 mg/l suspensions were centrifuged for 15 minutes at 2000x*g* to sediment the particle aggregates. The supernatant was used for NOM and phosphate analysis using methods described above. Concentrations were expressed relative to the identically treated control.

3. RESULTS

3.1. Nanoparticle characterization

The average diameters of SiO_2 and alumina coated SiO_2 NPs of original stock dilutions in deionized water at the same pH of the original stock and of dialyzed stock dilutions in OECD medium at various pH values and natural organic matter (NOM)

concentrations are given in **Table 1**. The SiO_2 NPs were stable in deionized water at pH 9.6, since the diameter obtained in the DLS measurements corresponded well with the BET primary particle size of 22 nm. The average DLS diameters around 31 nm obtained in OECD medium suggested that some aggregation occurred under test conditions. The alumina coated SiO_2 NPs formed small aggregates in deionized water at pH 4, where a mean diameter of 39 nm was obtained. In all other media, severe aggregation gave rise to micrometer size particles, of which the actual size was too large to be accurately determined by dynamic light scattering. The addition of NOM did not affect the stability of the silica NP suspensions. The zetapotential values varied with pH, as reported in **Table 2**. For both particles, the surface charge became more negative at higher pH values. However, the SiO_2 NPs having a zetapotential between -23 and -47 mV were negatively charged over the entire pH interval used in this study, while the zetapotential of the alumina coated SiO_2 NPs switched from positive to negative between pH 7.5 and 9.0. Those observations are fully in line with the reported IEP values of SiO_2 and Al_2O_3, respectively.

3.2. Algal growth inhibition assessments

First, it was investigated if dialyzing the NP suspensions affected their toxicity. The concentration-response curves of dialyzed and non dialyzed SiO_2 NPs collapsed, regardless of the algal cell density measurement method. **Figure 1**A presents those concentration-response curves. However, in case algal cell density was analyzed by fluorescence of extracted chlorophyll, the effect on growth rate was much more severe compared to the cell counting method. For example, the highest test concentration of 460 mg/l resulted in 56 % or 92 % decrease in growth rate when assessed using cell counting or extracted chlorophyll fluorescence, respectively. A different outcome was obtained when comparing concentration-response curves of dialyzed and non dialyzed alumina coated SiO_2 NPs. Cell

253 counting resulted in non-monotonous concentration-response curves, with apparently high
254 toxicity at low NP concentrations. However, clusters of algal cells growing in flocs could
255 be visually observed, as illustrated in **Figure 2.** Hence, the measured cell count was much
256 lower compared to the real density of individual cells of which the clusters were composed.
257 Chlorophyll fluorescence analysis resulted in monotonous concentration-response curves,
258 with the non-dialyzed suspension causing a more severe effect on algal growth rate
259 compared to the dialyzed NP suspension. At the highest test concentration 55 % decrease
260 was observed for non-dialyzed suspensions against only 25 % for dialyzed suspensions.
261 Possibly, impurities present in LUDOX CL can partly explain the observed effects of non
262 dilayzed suspension. From Figure S1 in supporting information it can be observed that
263 more impurities were removed from the LUDOX CL nanoparticle suspensions during the
264 dialysis compared to the LUDOX CL-X nanoparticle suspensions. The four concentration-
265 response curves are given in **Figure 1**B. An overview of 48 h 10, 20 and 50 % effect
266 concentrations on growth rate, as well as No Observed Effect Concentrations (NOECs) and
267 Lowest Observed Effect Concentrations (LOECs) are given in **Table 3**. The 72 h values are
268 reported in **Table S1** in the supplementary information. For all subsequent experiments the
269 dialyzed suspensions were used.

270 The concentration-response curves with concentration both expressed as mass and
271 as surface area are given in **Figure S2**.

272 Secondly, the toxicity of both NPs with varying pH was assessed. All curves
273 obtained using fluorescence of extracted chlorophyll are given in **Figures 3**A and 3B. The
274 concentration-response curves obtained using the cell counter are shown in the
275 supplementary information in **Figure S3**A. At the highest test concentration of 220 mg/l no
276 significant reduction in algal growth rate was observed for SiO_2 NPs at pH 6.0. At pH 6.8
277 and 8.6, concentration-response curves were similar, for which 10 % effect concentrations

(E_rC10) of 12.3 and 13.2 mg/l were established, respectively. The highest toxicity was observed at pH 7.6, with 48 h-E_rC10 of 6.1 mg/l. The alumina coated SiO_2 NPs showed a different pH influence on toxicity (**Figure 3B**). At low test concentrations, no clear pH effect was observed. At the higher test concentrations, pH 8.6 resulted in the lowest toxicity with a NOEC of 100 mg/l. At pH values 6.0, 6.8 and 7.6, the NOECs were 4.6 mg/l. The 52 % decrease in algal growth rate in the highest test concentration at pH 6.0 was the only exposure condition causing an effect > 50 %. The 48 h effect concentrations, NOECs and LOECs can be found in **Table 4**. Concentration-response curves obtained using cell counting and corresponding effect parameters are given in the supplementary information in **Figure S3**B and **Table S2**.

In addition, the relation between toxicity and specific surface area of both particles can be evaluated. Based on the specific surface area measurements (203 m^2/g for the alumina coated SiO_2 NPs and 102 m^2/g for the SiO_2 NPs) only, the alumina coated SiO_2 NPs were expected to be most toxic. However, from **Tables 3 and 4** it is clear that the difference in toxicity cannot be explained by surface area. For example, at pH values 6.8-8.6 48 h-E_rC_{20}s of SiO_2 NPs ranged between 9.9 and 20.4 mg/l, while those of alumina coated SiO_2 NPs were higher and ranged between 25.2 and 342.1 mg/l. This demonstrates that here, in case of different surface chemistry, toxicity was not governed by specific surface area only, and that coating characteristics can dominate toxic response.

Finally, the influence of natural organic matter (NOM) on the toxicity of both NP types was assessed at pH 7.4. Addition of NOM to the test medium before spiking the NPs decreased their toxicity. For SiO_2 NPs, 48 h-E_rC10 values were 25.6, 120.6 and 263.5 mg/l in presence of 1.2, 4.7 and 9.0 mg C/l NOM, respectively, which represents a 2-19 fold increase. All ecotoxicological effect parameters are summarized in **Table 5**. No toxicity was observed at the highest test concentration of 1000 mg/l alumina coated SiO_2 NPs in

303 presence of any concentration of NOM. **Figure 4**A and 4B show all concentration-response
304 curves assessed using chlorophyll detection. In **Table S1, Table S2 and Figure S4** of the
305 supplementary information concentration-response curves based on cell counting and their
306 corresponding 48 h effect parameters, as well as 72 h effect parameters are included.

307 The 96 well plate experiments to assess the shading effect indicated that toxicity
308 was not due to light limitation. When algal cells and NP suspensions were spatially
309 separated, no decrease in chlorophyll contents relative to the control was observed.
310 However, when algal cells were directly exposed to the NP suspensions, chlorophyll
311 contents decreased with >80 % relative to the control. The results are graphically shown in
312 **Figure 5**.

313 **3.3. Interactions between nanoparticles and test medium**

314 First, the Vivaspin 20 filter performance was checked for retaining or leaching of
315 analytes. In four filtering processes with OECD medium no phosphate or ammonium was
316 retained by the filters. Mean (std. dev.) recoveries of 100.8 (2.4) and 100.2 (0.7) % were
317 obtained, respectively. On the other hand, leachage of these nutrients into filtered deionized
318 water was negligible because absorbance values were not significantly higher than those of
319 deionized water blanks that were not forced through the filters. The concentration of
320 dissolved silica in filtered OECD medium and deionized water run through a particle
321 containing filter was beneath the detection level of the colorimetric method (< 0.050 mg/l).
322 Finally, after filtering a 0.5 mg/l Al standard in OECD medium at pH 2, a recovery of 90.4
323 (9.8) % was obtained.

324 The SiO_2 NPs partially dissolved in OECD medium and their solubility was pH
325 dependent, as presented in **Table 6**. At pH 8.6, 68.6 mg SiO_2/l reactive silica was present in
326 the highest SiO_2 NP concentration of 460 mg/l, which is a reactive silica concentration 11
327 times higher compared to the same suspension at pH 6.0. On the other hand, the reactive

328 silica concentration in the alumina coated SiO_2 NP suspensions was low. At the highest NP
329 concentration of 460 mg/l, the reactive silica concentration was not higher than 1.1 mg/l as
330 SiO_2. Only in the filtrate of the 460 mg/l suspension at pH 8.6 a significant amount of
331 aluminum was detected (0.068 mg/l or $2.5x10^{-3}$ mM). However, the chemical speciation
332 program Visual MINTEQ indicated that only $3.12x10^{-5}$ mM ionic Al can be present in
333 OECD medium at pH 8.6. Any excess of Al was predicted to precipitate as the mineral
334 diaspore (α-AlO(OH)). On the other hand, Parent et al. (1996) reported 30 % reduction in
335 algal growth rate of the green alga *Chlorella pyrenoidosa* exposed to 6.2 µM mononuclear
336 inorganic Al for 96 h. As a consequence, it is not likely that the decrease in algal growth
337 rate during exposure to alumina coated SiO_2 NPs was caused by the presence of inorganic
338 mononuclear dissolved Al. However, very small (< 10 kDa) secondary diaspore colloids in
339 460 mg/l suspensions at pH 8.6 could have passed the ultrafilter. Also, aluminum is known
340 to form polynuclear compounds (Parent et al., 1994). Such colloids and/or polynuclear
341 compounds could have been formed after partial dissolution of the $Al(OH)_3$ coating and
342 desorption of Al^{3+} and/or its dissolved hydroxides. Hence, it cannot be excluded that these
343 small secondary colloids did not contribute to the observed toxic effects.

344 Colorimetric Al analysis of 0.5 mg/l standards in OECD medium (without Fe and EDTA)
345 at various pH values before and after ultrafiltration experimentally confirmed the predicted
346 precipitation of the Visual MINTEQ program. Indeed, at pH 4.5, 7.6 and 11.0, recoveries
347 (std. dev. on the mean of five replicates) of 3.3 (3.6), 15.0 (14.4) and -0.1 (3.7) % were
348 obtained, which allowed to conclude that Al precipitated and did not pass the ultrafilter. On
349 the other hand, 48 h-NOECs ≥ 100 µg/l (3.7 µM) were established for the (precipitated)
350 alumina standard tested in the pH range of 6.0-8.6. The lowest NOEC of 100 µg/l was
351 observed at pH 6.8, while the highest NOEC of 460 µg/l was observed at pH 7.6 and 8.6. At
352 the highest test concentration of 2200 µg/l total Al, toxicity was highest at pH 6.0 and 6.8.

The latter toxicity parameters correspond well to the 96 h-E_rC_{50} of 576 µg/l total Al established by Call et al. (1984) for *Pseudokirchneriella subcapitata* at pH 7.25-7.89. This indicates that Al toxicity was strongly influenced by its pH dependent speciation, as described previously by Klöppel et al. (1997). Still, since it is currently unclear to what extent the Al desorbed from the alumina coated SiO_2 NPs and subsequently precipitated, it cannot be excluded from these experiments that secondary colloids contributed to the decrease in algal growth rate. In the supplementary information section **Table S3** reports the Al speciation of a 0.5 mg/l Al solution in OECD medium at various pH values. **Figure S5** shows the concentration-response curves obtained for an Al standard in OECD medium at pH 6.0-8.6.

Ammonium did not adsorb to the surface of both NP types. Phosphate, on the other hand, did not adsorb to the SiO_2 NPs, but showed a strong pH dependent affinity towards the alumina coated SiO_2 NP surface, with increased sorption at low pH. At pH 6.0 and 6.8, a NP concentration of 460 mg/l decreased the available phosphate concentration to a value below the detection level of the colorimetric analysis (0.03 mg PO_4^{3-}/l), which meant that more than 97.3 % of the phosphate was adsorbed to the NP surface. The concentration of available phosphate in function of pH and alumina coated SiO_2 NP concentration is presented in **Figure 6**.

The concentration of NOM in centrifuged 460 mg/l alumina coated SiO_2 NP suspensions was significantly lower compared to the NP free control for all three NOM concentrations. At lower NP concentrations, no significant decrease was detected. At NOM concentrations of 2.7, 7.4 and 12.5 mg C/l, the decrease in NOM concentration (std. dev., n = 2) was 1.3 (0.1), 3.2 (0.2) and 4.3 (0.1) mg C/l, respectively. This corresponds to an adsorption of NOM to the NP surface (std. dev., n = 2) of $2.7x10^{-3}$ ($0.3x10^{-3}$), $7.0x10^{-3}$

377 ($0.5x10^{-3}$) and $9.3x10^{-3}$ ($0.3x10^{-3}$) mg C/mg NP. **Figure S6** in the supplementary
378 information summarizes the measured concentrations in all suspensions.

379 In the 7.4 and 12.5 mg C/l NOM suspensions, the phosphate adsorption was lower
380 compared to the NOM free medium. For example, in absence of NOM, 460 mg/l alumina
381 coated SiO_2 NPs adsorbed (mean (std.dev.) of 2 replicates) 93.1 (4.5) % of total phosphate.
382 However, in presence of 7.4 and 12.5 mg C/l NOM those suspensions adsorbed only 76.8
383 (1.5) and 66.9 (1.4) %, respectively. **Table S4** in the supplementary information gives an
384 overview of all phosphate measurements in function of NOM concentration.

385 **4. DISCUSSION**

386 Because of the identical composition of the core material, both particles are sold as
387 a colloidal silica suspension. However, from the characterization data, it is clear that the
388 alumina coating completely changed the particle surface characteristics and stability
389 behaviour. Consequently, our results emphasize the importance of detailed product
390 specification in nanoparticle containing products and in nanoparticle studies. The difference
391 in stability in the OECD medium can be explained using the zetapotential measurements.
392 Upon lowering the pH to 6.0, the surface charge on the bare silicon dioxide particles
393 became less negative, though was still large enough to prevent aggregation. Only at pH
394 values < 2.5 the SiO_2 NPs were expected to bare no charge (Van der Meeren et al., 2004).
395 Due to the low surface charge on the alumina coated SiO_2 NPs, the suspensions in OECD
396 medium were unstable and particles aggregated severely. The zetapotential measurements
397 suggested a point of zero charge between pH 7.5 and 9.0, which was in agreement with the
398 studies performed by Van der Meeren et al. (2004) and Jiang et al. (2009) in which iso-
399 electric points of 8-8.5 were obtained.

400 The bare SiO_2 NPs were more toxic to the alga compared to the alumina coated
401 SiO_2 NPs, despite the fact that the alumina coated SiO_2 NPs presented a larger amount of

specific surface area. Apparently, the alumina coating interacted differently with the alga. As a consequence, the relation between toxicity and surface area, that was demonstrated for 12.5 and 27.0 nm SiO_2 NPs (Van Hoecke et al., 2008), was no longer valid when NPs bore a chemically different coating on their surface. Again, it is clear that the surface dominated NP characteristics. Hence, from a risk assessment point of view, NP coatings should be taken into account, whereby physical and chemical as well as toxicological characteristics of coated NPs cannot be directly extrapolated from knowledge on non-coated NPs. However, since only one organism was used in the present study, care must be taken not to generalize the higher toxicity of alumina coated silica NPs. More experimental data on other organisms is to be obtained. Nevertheless, an in vitro study with bare and alumina coated LUDOX® silica NPs also showed lower cytotoxicity, reactive oxygen species and DNA double strand breaks when a human neuronal cell line was exposed to the alumina coated NPs compared to the bare silica particles (Kim et al., 2010).

Chlorophyll analysis of algae exposed to the SiO_2 NPs indicated a much more severe effect on algal growth rate compared to the cell count based data. In fact, at the highest test concentration, the algal cells almost completely lacked chlorophyll. The fluorescent signal in the 460 mg/l sample was 96 % lower compared to the control and at 46 mg/l 82 % decrease was found. These results therefore suggest that breakdown of chlorophyll and/or chlorophyll synthesis inhibition was an important aspect of the mechanistic toxic response induced by the SiO_2 NPs. A similar conclusion was also drawn by Wei et al. (2010) who observed between 75 and 95 % decrease in chlorophyll upon exposure of the alga *Scenedesmus obliquus* to silica NPs at 50 to 200 mg/l. Due to the clustering between algal cells and alumina coated SiO_2 NPs and the resulting erroneously low cell density measurement, it was not possible to draw conclusions on a similar direct effect of these NPs on chlorophyll.

The clustering of algal cells and alumina coated SiO_2 NPs was caused by the electrostatic attraction between the positively charged particles and the negatively charged algal cells. The alumina coated SiO_2 NP aggregates acted as a binding agent between algal cells. An identical observation was made by Jiang et al. (2009) and Simon-Deckers et al. (2009) upon exposure of bacteria to Al_2O_3 NPs. Both articles mention the flocculation of cell suspensions in presence of Al_2O_3 NPs. In view of the fact that *Pseudokirchneriella subcapitata* naturally occur as singular cells (Nygaard et al., 1986), their ability to survive and grow in a clustered structure is remarkable. However, from an ecological point of view the cluster formation can indirectly affect the algal community and species at higher levels of the aquatic ecosystem. At one hand, increased gravity of clusters of algal cells and particle aggregates can enhance their sedimentation to deeper levels of the surface water body, where light intensity is lower. This way, the cluster formation can indirectly affect algal growth (Navarro et al., 2008). Due to the decreased availability of algal cells in the pelagic part of the aquatic environment, species higher up the aquatic food chain may suffer from food limitation. In a previous study, we experimentally confirmed this mode of action principle for CeO_2 NP aggregates in chronic *Daphnia magna* survival and reproduction tests (Van Hoecke et al., 2009). Due to the large negative surface charge of the SiO_2 NPs, a similar clustering with algal cells was not expected and also not observed. Nevertheless, the SiO_2 NPs were able to interact directly with the algal cells through adsorption to the cell wall, as investigated in a previous study with similar LUDOX SiO_2 NP suspensions (Van Hoecke et al., 2008).

It turned out that NP toxicity can be strongly pH dependent. In general, except at pH 6.0, the bare SiO_2 NPs were more toxic compared to the alumina coated ones. However, it is currently not clear why no toxic effect was observed for the SiO_2 NPs at pH 6.0. Possibly, the decreased surface charge diminished NP reactivity. Again, the large impact of

452 the alumina coating on SiO_2 NP characteristics was apparent from the algal growth
453 inhibition tests assessed at various pH values. Alumina coated SiO_2 NPs were shown to be
454 most toxic at pH 6.0 and 6.8 and least toxic at pH 8.6. Possibly, the pH dependent
455 phosphate adsorption contributed to the differential toxicity (**Figure 6**). Indeed, since no
456 phosphate was available at high test concentrations at pH 6.0 and 6.8, the algae could have
457 suffered from a lack of nutrients. Previously, it was shown that decrease in algal growth
458 rate occurred up to 50 % if no phosphate was available (Van Hoecke et al., 2009). The
459 chemical analysis data furthermore indicated that dissolution of the bare SiO_2 NPs was
460 substantial. This is not surprising, since the solubility of amorphous silica in water at 25 ° C
461 is 120 mg/l (Alexander et al., 1954). Hence, during the algal growth inhibition tests, no
462 equilibrium was reached between solid and dissolved silica. On the other hand, dissolution
463 of the silica core of alumina coated NPs was inhibited due to the presence of the coating.
464 The chemical analysis data furthermore indicated that dissolution of the silica core is
465 inhibited due to the $Al(OH)_3$ coating. In addition to the knowledge that dissolved Al species
466 precipitate from $0.03x10^{-3}$ to $0.8x10^{-3}$ mg/l on, it is not likely that toxicity of alumina
467 coated SiO_2 NPs was caused by the presence of dissolved aluminum species either.
468 Similarly, Jiang et al. (2009) concluded that bacterial toxicity of Al_2O_3 particles at 20 mg/l
469 was not due to dissolution, since concentrations were below the ICP-OES detection level.
470 The addition of NOM to the test media strongly decreased toxicity. Since the NOM
471 was able to adsorb to the alumina surface, the decrease in toxicity could be due to the
472 shielding of the NPs by NOM, preventing a direct interaction with algal cells, which caused
473 a decrease in bioavailability. Yang et al. (2009) experimentally demonstrated the adsorption
474 of humic acid, a component of NOM, to the Al_2O_3 NP surface and attributed this behaviour
475 to the NPs' large surface area, low hydrophilicity, few negative charges and the strong
476 physical interactions between humic acid and the NP surface, like electrostatic attraction

and ligand exchange. When analyzed using a cell counter, concentration-response curves of alumina coated SiO_2 NPs were, again, different from those assessed using fluorescent detection of extracted chlorophyll, as shown in **Figure S3**. For SiO_2 NPs the effect concentrations in presence of NOM, listed in **Table 5**, were found to increase linearly with increasing NOM concentration, which is illustrated in **Figure 7.** In **Figure S7**, the 72 h toxicity data of these tests are also presented. **Table S5** lists the a, b and determination coefficient (R^2) parameters of the linear regressions, calculated using least squares analysis. However, to date it is not clear through which mechanism the NOM decreased SiO_2 NP toxicity. Unlike for the alumina coated SiO_2 NPs, it was difficult to separate the bare SiO_2 NPs from the NOM due to the colloidal stability of the suspension. Consequently, the NOM adsorption to SiO_2 NPs could not be quantified. Humic acid, one of the components of NOM, was not found to adsorb to SiO_2 NPs, which was explained through the high hydrophilic nature of the SiO_2 surface (Yang et al., 2009). However, other compounds of NOM can still adsorb to the SiO_2 NPs. For example, acid-base interactions between organic acid groups in NOM and hydroxyl groups on the silica surface can establish the adsorption (Considine et al., 2005). In addition, NOM is also able to bind to algal cells, which can in turn shield the algal cell surface from direct interaction with NPs. Campbell et al. (1997) demonstrated the binding of dissolved organic matter to the cell surface of phytoplankton. The authors suggested that either a hydrogen-bonding sorption mechanism between negatively charged functional groups in the DOM and on the cell surface or the formation of hydrophobic bonds between both are involved.

In conclusion, the assessment of particle-particle, particle-test medium and particle-alga interactions confirmed that the application of a thin layer of alumina onto the surface of SiO_2 NPs completely altered their characteristics. First, due to the low surface charge, alumina coated SiO_2 NPs aggregated in test medium, while bare SiO_2 NPs were stable.

Second, bare SiO_2 NPs were more toxic in standard OECD test medium and showed a different pH dependent toxicity compared to the alumina coated ones. Third, dissolution and nutrient adsorption characteristics were different. As a consequence, coating formulations should be taken into account when performing risk assessments of engineered NPs.

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628 **TABLES**

629 **Table 1** – Average particle diameters (Zave) and polydispersity indices (PI) obtained with

630 dynamic light scattering in various media

631

Product	**Medium**	**pH**	**Zave (nm) (std.dev.)**	**PI (std. dev.)**
SiO_2 NPs (LUDOX CL-X)	Deionized water	9.6	20.9 (0.1)	0.26 (0.01)
	OECD	6.0	33.3 (0.5)	0.09 (0.02)
	OECD	6.8	32.3 (1.0)	0.13 (0.10)
	OECD	7.6	31.4 (2.3)	0.19 (0.12)
	OECD	8.6	31.9 (2.2)	0.14 (0.13)
	OECD + 2 mg C/l NOM	7.4	32.7 (0.6)	0.10 (0.03)
	OECD + 6 mg C/l NOM	7.4	32.6 (1.6)	0.14 (0.08)
	OECD + 10 mg C/l NOM	7.4	32.9 (1.1)	0.12 (0.10)
$Al(OH)_3$ coated SiO_2 NPs (LUDOX CL)	Deionized water	3.7	39.4 (1.2)	0.24 (0.01)

632 **Table 2** – Zetapotential values of SiO_2 and alumina coated SiO_2 NPs in various media

Product	Medium	pH	Zetapotential (mV) (std.dev.)
SiO_2 NPs (LUDOX CL-X)	OECD	6.0	-23.1 (0.4)
	OECD	7.5	-32.8 (1.1)
	OECD	9.0	-37.7 (5.5)
	Deionized water	9.6	-47.3 (5.2)
$Al(OH)_3$ coated SiO_2 NPs (LUDOX CL)	Deionized water	3.7	39.6 (3.2)
	OECD	6.0	20.6 (0.4)
	OECD	7.5	4.9 (0.5)
	OECD	9.0	-6.7 (0.2)

633

634 **Table 3 -** NOECs, LOECs and 48 h-E_rC_x (95 % conficence interval) values of dialyzed and
635 non dialyzed NP suspensions at pH 7.4 in standard OECD algal test medium. Both cell
636 counting (CC) and fluorescence of extracted chlorophyll (Chl) were used to determine algal
637 cell density.
638

Particle	**CC/ Chl**	**NOEC**	**LOEC**	**E_rC_{10}**	**E_rC_{20}**	**E_rC_{50}**
		mg/l	mg/l	mg/l	mg/l	mg/l
SiO_2 non dialyzed	CC	22	46	26.9 21.7-33.4	36.7 32.1-42.1	n.d.
	Chl	22	46	18.2 14.4-23.0	26.0 21.9-30.9	48.0 43.4-53.1
alumina coated SiO_2 non dialyzed	CC	< 10	< 10	< 10	< 10	< 10
	Chl	10	22	> 460	14.2 9.3-21.6	35.1 26.6-46.1
SiO_2 dialyzed	CC	22	46	28.3 22.0-36.5	36.7 31.5-42.9	n.d.
	Chl	22	46	14.0 11.1-17.8	21.8 18.3-26.1	46.4 41.9-51.5
alumina coated SiO_2 dialyzed	CC	< 10	10	< 10	< 10	< 10
	Chl	22	46	46.2 33.7-63.3	120.9 88.6-165.0	> 460

639 **Table 4** - Ecotoxicological effect parameters (95 % confidence interval) of algal growth
640 inhibition assays with SiO_2 and alumina coated SiO_2 NPs in standard OECD algal test
641 medium at various pH values, analyzed after 48 h. Algal cell density was analyzed using
642 fluorescent detection of extracted chlorophyll.

Particle	**pH**	**NOEC** mg/l	**LOEC** mg/l	**E_rC_{10}** mg/l	**E_rC_{20}** mg/l	**E_rC_{50}** mg/l
SiO_2 dialyzed	6.0	n.d.	> 220	> 220	> 220	> 220
	6.8	10	22	12.3 8.5-17.7	20.4 16.0-25.9	58.6 49.1-69.9
	7.6	4.6	10	6.1 4.6-8.1	9.9 8.1-12.1	25.7 22.7-29.1
	8.6	10	22	13.2 10.5-16.6	19.2 16.4-22.4	42.2 37.2-47.7
alumina coated SiO_2 dialyzed	6.0	4.6	10	47.5 26.8-84.0	118.8 83.5-169.1	n.d.
	6.8	4.6	10	9.5 5.3-16.8	25.2 16.5-38.4	n.d.
	7.6	4.6	10	12.9 4.2-40.0	155.7 70.3-344.7	n.d.
	8.6	100	220	179.2 129.1-248.6	342.1 290.3-403.1	n.d.

643 **Table 5** - NOECs, LOECs and E_rC_x values (95 % confidence interval) of both NPs types in
644 OECD algal test medium at pH 7.4, in presence of various NOM concentrations. Algal cell
645 density was analyzed using fluorescence of extracted chlorophyll.

Particle	**NOM**	**NOEC**	**LOEC**	**E_rC_{10}**	**E_rC_{20}**	**E_rC_{50}**
	mg C/l	mg/l	mg/l	mg/l	mg/l	mg/l
Dilayzed SiO_2 NPs	1.2	22	46	25.6 18.4-35.6	55.8 43.6-71.4	211.9 185.0-242.6
	4.7	100	220	120.6 100.3-145.0	218.9 192.4-249.0	606.6 567.2-648.7
	9.0	100	220	263.5 232.7-298.3	462.0 423.1-504.5	1206.9 1151.7-1264.7
Dialyzed alumina coated SiO_2 NPs	1.3	> 1000	> 1000	> 1000	> 1000	> 1000
	4.9	> 1000	> 1000	> 1000	> 1000	> 1000
	9.1	> 1000	> 1000	> 1000	> 1000	> 1000

646 **Table 6 –** Concentration of reactive (dissolved) silica in SiO_2 NP suspensions in OECD
647 medium at various pH values, assessed after 48 h incubation under test conditions. Standard
648 deviation on two replicated measurements are given in between parentheses. DL =
649 detection limit = 0.050 mg SiO_2/l.

Conc. SiO_2 NPs	**Concentration of dissolved silica (mg SiO_2/l)**			
(mg/l)	**pH 6.0**	**pH 6.8**	**pH 7.6**	**pH 8.6**
4.6	< DL	< DL	< DL	< DL
46	< DL	1.8 (0.3)	2.9 (0.0)	18.1 (0.3)
460	6.0 (0.4)	12.6 (0.3)	26.5 (0.2)	68.6 (0.3)

Figure legends

Figure 1 – Concentration-response curves of SiO_2 and $Al(OH)_3$ coated SiO_2 NPs in standard OECD test medium at pH 7.4. Algal density was measured using cell counting (CC) and using fluorescence of extracted chlorophyll (Chl). Algal growth rate was expressed relative to the control (% rtc) and error bars represent standard deviation on the mean growth rate (n = 3).

Figure 2 - Algae clusters in 10 mg/l alumina coated SiO_2 NPs on 3rd day of algal growth inhibition test.

Figure 3 – Concentration-response curves of SiO_2 and alumina coated SiO_2 NPs in OECD medium at various pH values. Algal cell density was analyzed using chlorophyll fluorescence. Error bars represent standard deviation on mean growth rate calculated after 48h.

Figure 4 – Concentration-response curves of SiO_2 and alumina coated SiO_2 NPs in OECD medium at pH 7.4 in presence of various natural organic matter concentrations. Algal cell density was analyzed using chlorophyll fluorescence. Error bars represent standard deviation on mean growth rate.

Figure 5 – 96 well plate experiment to assess the importance of light limitation. Algal cells were always spiked into the lower white plate. In the control, both the white and opaque plate contained standard OECD test medium. When algae and NPs were spatially separated, the white plate contained OECD medium while NPs in OECD medium were spiked into the opaque plate. Treatmens where algal cells and NPs were in direct contact, the white plate contained both algal cells and NPs in OECD medium, while the opaque plate contained OECD medium only.

Figure 6 – Concentration of phosphate in ultrafiltered OECD medium in function of alumina coated SiO_2 NP concentration for various pH values, expressed as % relative to the standard OECD medium (% rtc).

Figure 7 – Illustration of linear relations between the NOM content of OECD medium and the established effect concentrations of SiO_2 NPs on algal growth rate, assessed after 48 h using chlorophyll analysis.

Figure 1

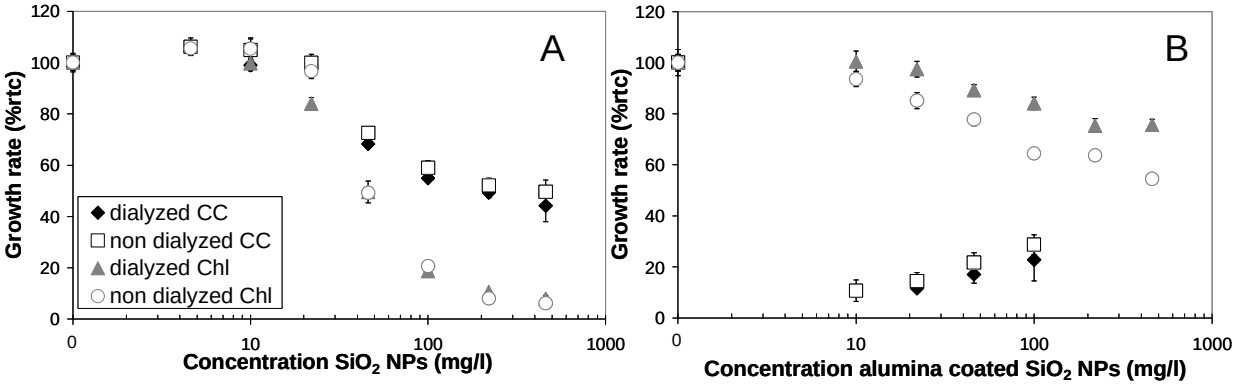
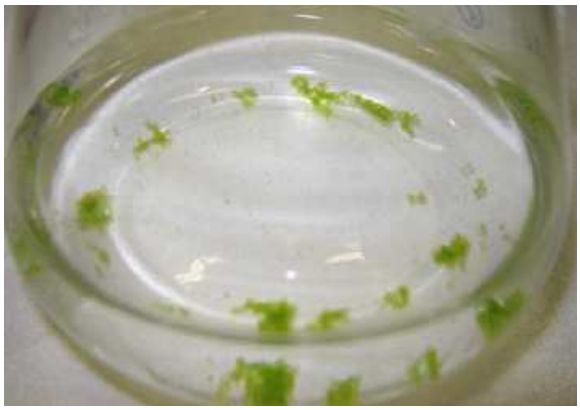

A
B
Growth rate (%rtc)
dialyzed CC
non dialyzed CC
dialyzed Chl
non dialyzed Chl
Concentration SiO2 NPs (mg/l)
Concentration alumina coated SiO2 NPs (mg/l)
0
20
40
60
80
100
120
10
1000

Figure 2

Color for publishing on the web :



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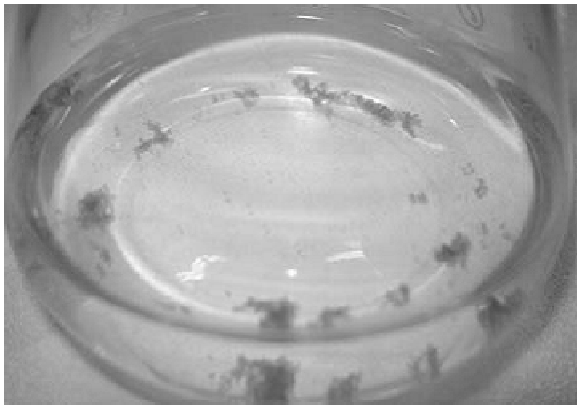


Figure 3

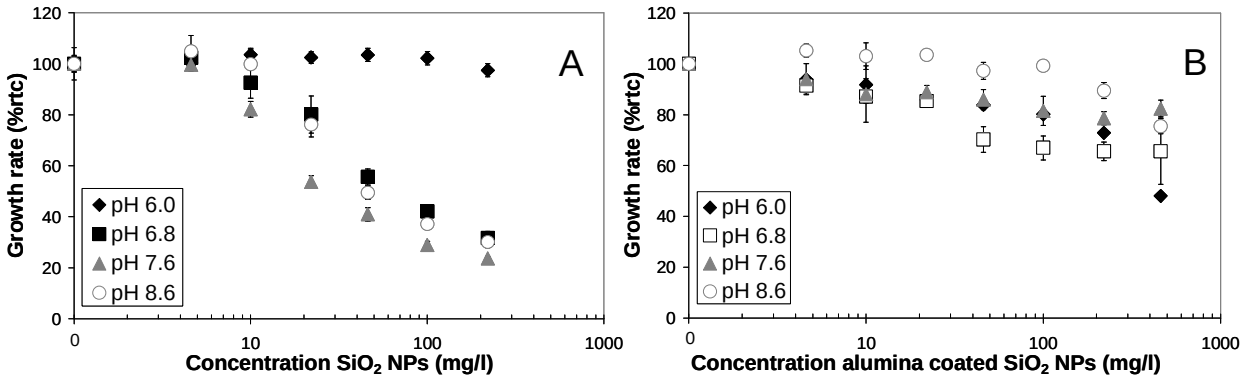

A
B
Growth rate (%rtc)
pH 6.0
pH 6.8
pH 7.6
pH 8.6
Concentration SiO_2 NPs (mg/l)
Concentration alumina coated SiO_2 NPs (mg/l)
0
20
40
60
80
100
120
10
100
1000

Figure 4

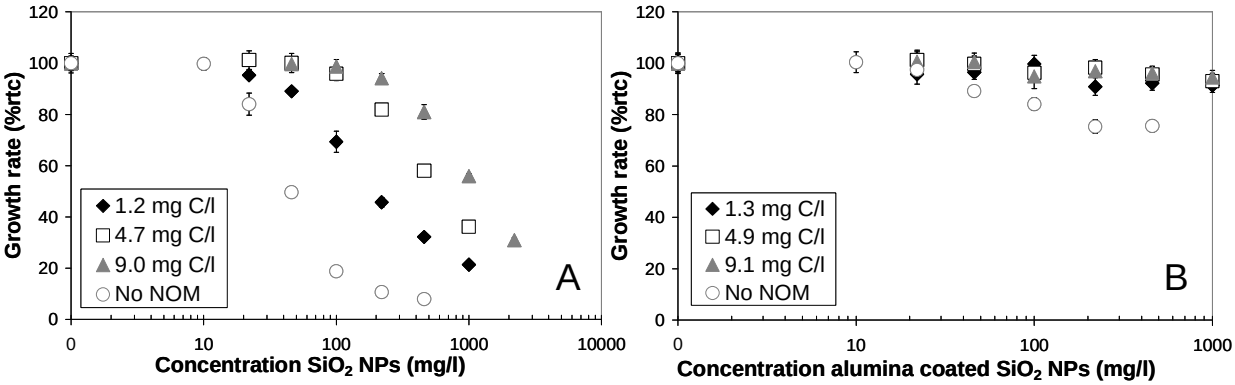

Growth rate (%rtc)
120
100
80
60
40
20
0
0
10
100
1000
10000
Concentration SiO2 NPs (mg/l)
1.2 mg C/l
4.7 mg C/l
9.0 mg C/l
No NOM
A
Growth rate (%rtc)
120
100
80
60
40
20
0
0
10
100
1000
Concentration alumina coated SiO2 NPs (mg/l)
1.3 mg C/l
4.9 mg C/l
9.1 mg C/l
No NOM
B

Figure 5

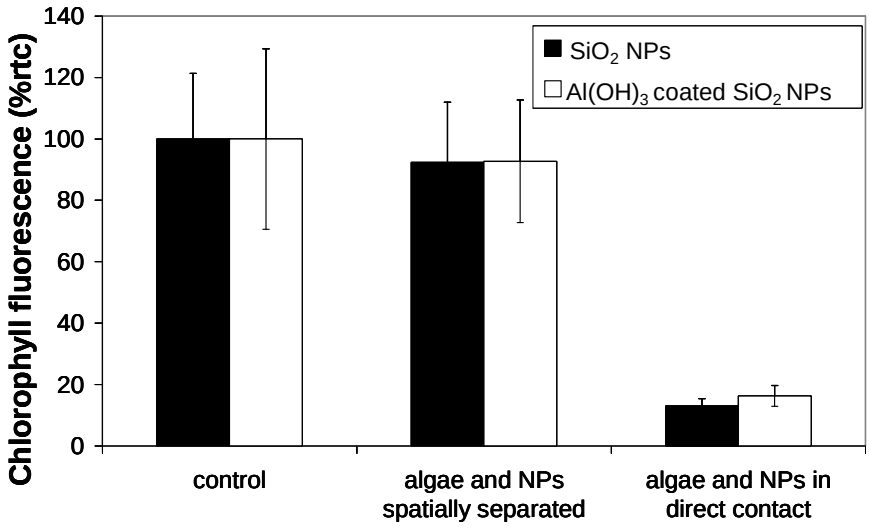

Chlorophyll fluorescence (%rtc)
140
120
100
80
60
40
20
0
SiO2 NPs
Al(OH)3 coated SiO2 NPs
control
algae and NPs spatially separated
algae and NPs in direct contact

Figure 6

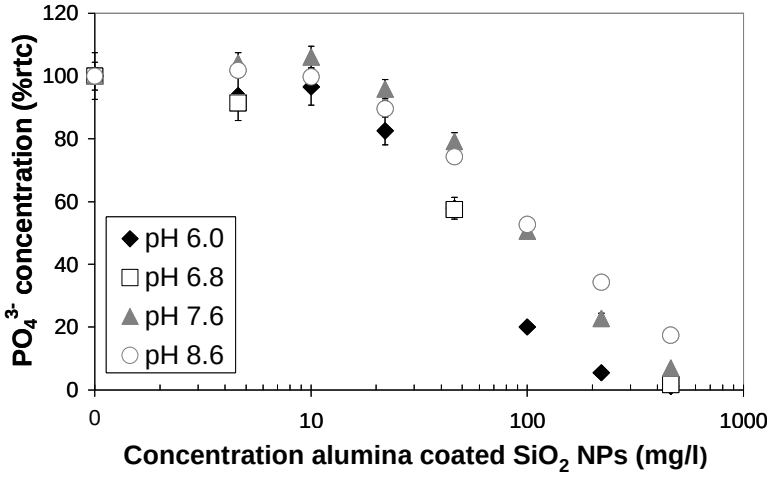
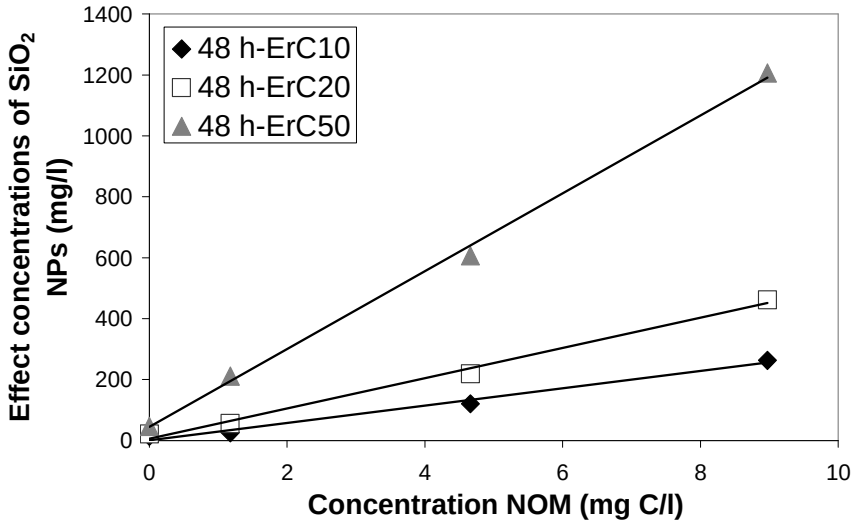

120
100
80
60
40
20
0
0
10
100
1000
PO4 3- concentration (%rtc)
Concentration alumina coated SiO2 NPs (mg/l)
pH 6.0
pH 6.8
pH 7.6
pH 8.6

Figure 7


48 h-ErC10
48 h-ErC20
48 h-ErC50
Effect concentrations of SiO2 NPs (mg/l)
Concentration NOM (mg C/l)
0
200
400
600
800
1000
1200
1400
0
2
4
6
8
10